INDICATOR GRADIENT PATTERNS IN OÖCYTES AND EARLY DEVELOPMENTAL STAGES OF ECHINODERMS: A REEXAMINATION

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The gradient patterns, as observed by means of intracellular reduction of methylene blue and diazine green (Janus green), following staining by oxidized dyes were recorded for cleavage and later stages of the echinoids, *Strongylocentrotus purpuratus, S. franciscanus, Dendraster excentricus* and for the asteroid starfish, *Patiria miniata,* and for exogastrulae of these forms in earlier papers (Child, 1936a, 1936b). Later the pattern of the intracellular indophenol reaction during larval development of *Dendraster* was determined, together with some data on patterns of exogastrulae (Child, 1941). In these observations on indophenol pattern, extremely low concentrations of reagents were used so that the patterns became visible in living animals. Still later, data concerning the indophenol gradient patterns of *Patiria miniata* from the later ovarian oöcytes to larval stages, also with very low concentrations of reagents, appeared (Child, 1944).

However, intracellular oxidation of reduced dyes and of indophenol reagents has been found more satisfactory than reduction after staining in the study of gradient patterns, for reasons noted in the following section. The present paper is primarily concerned with new data concerning gradient pattern, as determined by intracellular oxidation of indicators in stages from early ovarian oöcytes through embryonic development to larval stages of certain echinoderms.

Material and Methods

Two echinoids, *Strongylocentrotus purpuratus* and *Dendraster excentricus,* and the asteroid starfish, *Patiria miniata,* have served as material. The data include stages from the early ovarian oöcytes to earlier larvae of the echinoids. As regards the starfish, they are merely supplementary to the earlier study of the indophenol reaction in this form (Child, 1944). For the two echinoids these new data on intracellular oxidation of indicators constitute a more complete survey of indicator patterns than earlier studies and render differentials more clearly visible, particularly in certain stages. Deep indebtedness to the Director and staff of the Hopkins Marine Laboratory of Pacific Grove for collecting material, providing facilities for its use and, in various cases, for transporting it to Palo Alto, is gratefully acknowledged. For fertilization and development at Palo Alto filtered sea water kept in darkness has been found satisfactory.

In the first studies of indicator patterns in early echinoderm development (Child, 1936a, 1936b), the material was stained by an oxidized redox dye and reduction was brought about by oxygen uptake of embryos and larvae sealed in a small volume

of water or, in some cases, of dye solution. The decrease in free oxygen content of the solution brought about intracellular reduction of the dye. Reduction under these conditions was very slow and uniform distribution of oxygen was provided for by frequent change of position of the sealed material in relation to gravity or with motile stages by swimming activities. Reducing agents which had been used in earlier indicator studies were highly toxic and might themselves retard or inhibit reduction differentially by their toxicity.

As indicator studies continued it became increasingly evident that intracellular oxidized dyes, with increase in intracellular concentration, might also retard or even inhibit intracellular reduction differentially. This retardation or inhibition occurred most rapidly in the regions which reduced most rapidly in uninjured material; *i.e.*, if the oxidized indicator attained a certain intracellular concentration it might decrease or even obliterate slight regional differentials of pattern in consequence of the differential susceptibility of different gradient levels to the dyes, particularly the more toxic dyes such as diazine green (Janus green). In some cases a gradient pattern has become reversed in direction by the differential retardation or inhibition of intracellular reduction. In use of reduced dye solutions for intracellular oxidation, the toxic reducing agents might alter gradient pattern before intracellular oxidation began.

These difficulties are almost entirely avoided by use of sodium hydrosulphite as a reducing agent, either for dye solutions to be used for intracellular oxidation or for intracellular reduction after staining by an oxidized dye. This agent is not appreciably toxic, even with hours of exposure to concentrations far above those required to reduce the indicators, either in solution externally or intracellular. Very small quantities of this agent reduce dye solutions in a few seconds and it can be used at once for intracellular oxidation or for intracellular reduction of material previously stained by an oxidized dye. In the present paper it is used primarily for intracellular oxidation of dyes. A few minute granules of hydrosulphite, less than a milligram, are sufficient to reduce completely one ml. of methylene blue or diazine green solution and various other dyes in concentrations found useful. Intracellular oxidation occurs within a few minutes without evidence of toxic effect until intracellular dye concentration becomes high. Care must be taken not to use an excess of hydrosulphite beyond that required to reduce the dye. Any considerable excess may retard or even prevent intracellular oxidation in consequence of too complete removal of oxygen. After intracellular oxidation addition of a little more hydrosulphite will result in differential intracellular reduction. In many organisms differential oxidation and reduction can be repeated without alteration of pattern. These methods of using sodium hydrosulphite, although noted in earlier papers, are described in detail here because their use on early stages of echinoderm development is regarded as a somewhat critical case near the lower limit of availability of redox dyes for rendering visible regional differentials of pattern. Intracellular oxidation from reduced dye solutions has been called for convenience primary oxidation in order to distinguish it from re-oxidation, following reduction.

Redox dyes used for intracellular oxidation, often with following reduction, were methylene blue in various concentrations from 1/50,000 to 1/20,000, diazine green from 1/100,000 to 1/50,000 and Nile blue sulphate 1/100,000. These were

used chiefly on *S. purpuratus* for comparison with the indophenol reaction; on *Dendraster* and *Patiria* the indophenol reaction was used almost entirely. Primary oxidation of the redox dyes and the intracellular oxidation of the indophenol reagents have made visible the same patterns, though in certain cases the indophenol reaction has shown the differential more distinctly than the dyes.

The indophenol or Nadi reaction, the intracellular formation of the deep blue indophenol from para-aminodimethylaniline (dimethyl-paraphenylene diamine) and α-naphthol, catalyzed by an oxidase, "indophenol oxidase," commonly regarded as cytochrome oxidase or a related enzyme, has been found extremely useful in the studies of echinoderm pattern and in many other organisms. In several earlier papers the modifications of the reaction found most useful in rendering visible the slight differentials of early developmental stages and small single cells have been described. However, since presence of a gradient pattern in early stages of echinoderm development has been questiond, it has seemed necessary to call attention once more in some detail to the method, as used with low concentrations of reagents. Also certain points not particularly considered earlier are noted.

The aniline has been obtained in 10 gram amounts from the Eastman Kodak Co. Some of these have been liquid, others solid at room temperature and, according to information from the Kodak Co. laboratories, the solid form is more nearly pure. However, the melting point is only slightly above room temperature and immersion of the bottle containing the aniline in slightly warmed water liquefies it. Since the aniline is volatile and an acrid, highly irritating poison, weighing of the very small amounts required presents difficulties, and since earlier use of the aniline was in terms of small drops of the liquid, its use has been continued in this way. Although attempts have been made in earlier papers to give information concerning concentrations used, the actual concentrations are not known in any particular case and since the same pattern of reaction appears with a very wide range of concentrations and the method is not quantitative, the chief point at present is use of concentrations found by experience to give the reaction with as little indication of toxicity as possible. For most organisms and even for many single cells a solution, consisting of one small drop (30–40 = 1 ml.) in 10 ml. of salt or fresh water, according to the material, is made daily or oftener; the aniline gradually oxidizes in water. The concentration of this solution can be altered as desired. This is essentially a stock solution and is diluted for use. The α-naphthol stock solution consists of one mg. or less, estimated after repeated weighings as a basis for estimation, in 10 ml. of water, salt or fresh. Enough naphthol dissolves within a few minutes to give the reaction readily without addition of KOH or NaOH necessary to dissolve the naphthol when high concentrations of the agents are used.

The solution for use, regarded as standard, merely because it provides a starting point for determining the most satisfactory solution for a particular material, consists of one drop each of the aniline and the naphthol stock solutions in one ml. of water. The naphthol is much less toxic than the aniline and can be used in considerably higher concentrations if desired, but in any case it is not certain just how much of the naphthol dissolves without addition of alkali. In some organisms and with some cells concentrations half or a fourth of the standard, or even less, are desirable. Solutions four to five times the standard are usually rather rapidly toxic. In general the practice found most effective has been to use the lowest concentrations of reagents which render the intracellular reaction clearly visible within a

reasonable period of observation, *i.e.*, 10–30 minutes, although it is often possible to obtain a very distinct differential reaction in four to five minutes. The method is of course at present far from quantitative; its use in any case is a matter of trial and error, but it does render directly visible certain characteristics of physiological pattern in living organisms which are not now directly distinguishable in any other way, except with the method described above with redox dyes. With these general ranges of concentration and variations from them as occasion required, the indophenol reaction has made directly visible patterns of oxidase activity in numerous organisms from protozoa to vertebrates. At least the earlier stages of the reaction occur in apparently uninjured individuals; motile forms continue activity and non-motile developmental stages or forms may continue development until the intracellular concentration becomes high. With the much higher concentrations of the reagents, about 0.1 per cent and in some cases even one per cent, in some of the earlier work with the reaction and with alkali added to dissolve the α-naphthol, the material must have been killed almost at once, and little or no gradient pattern remained. For the echinoderm material twice the standard was commonly used, somewhat less often, the standard solution.

Some organisms apparently reduce or otherwise destroy intracellular indophenol with partial or complete loss of color, after the reaction has continued for a time, perhaps because of stimulation or irritation by the intracellular indophenol or the aniline. This decrease or loss of color has sometimes been observed in later echinoid blastulae and in gastrulae. Also, unless the intracellular concentration is very high there is usually more or less reduction with loss of color when cytolysis occurs. Intracellular reduction occurs rapidly on addition of very small amounts of sodium hydrosulphite, provided the intracellular concentration of indophenol has not become so high that reduction is retarded or inhibited. Re-oxidation and perhaps a second reduction are often possible without alteration of pattern.

The patterns of primary intracellular oxidation from dye solutions reduced by sodium hydrosulphite and of indophenol are similar but the indophenol reaction seems to show very slight differentials in the echinoderm material a little more distinctly than dye oxidation. Reduction patterns of the dyes and of indophenol are also similar. It is perhaps unnecessary to point out that the earlier stages of the indophenol reaction and also of primary dye oxidation are more important than the later stages in making visible the regional differentials of pattern. With increasing intracellular concentrations of oxidized dyes and of indophenol, slight differentials become less distinct or disappear as the color approaches uniformity. With low concentrations of dyes and indophenol reagents these methods are an exceedingly delicate means for showing directly slight regional differentials in activity of an oxidase or of oxidases and in reduction, of one or more dehydrogenases. They have an advantage over various other methods of not requiring separation of the organism into pieces.

In observation of these indicator patterns the most extreme precautions have been taken to avoid being deceived by apparent differentials resulting from direction or character of illumination or other extraneous factors. Frequent agitation has provided for uniform concentrations of dyes and indophenol reagents in non-motile stages. In motile stages the swimming activity of the animals serves this purpose. Single eggs have been moved about and turned over repeatedly. The patterns noted in this paper have been seen in hundreds of individuals during earlier and

later parts of the breeding seasons and in different lots of material during the last five years.

Figures are diagrammatic outlines with the gradients indicated by differential shading or by arrows pointing in the general direction of decrease in rate of intracellular oxidation, *i.e.,* down the gradient. Early cleavages of *S. purpuratus* and of *Dendraster excentricus* are alike. *Dendraster* eggs and cleavage stages are larger than those of the sea urchin but separate figures for these stages of the two species are regarded as unnecessary. The egg and developmental stages of the starfish, *Patiria,* are much larger than those of the echinoids and are outlined in the figures as slightly larger, but do not indicate the actual differences in size. In the shaded figures the shading is an attempt to indicate the differential as observed in particular cells, and nuclear position in the cell is drawn as observed. Arrows indicate merely the general directions of gradients, not their extent. Shorter arrows indicate less differential.

Growth Stages of Ovarian Oöcytes

In the earlier study of intracellular reduction of redox dyes in the ovarian oöcytes of *S. purpuratus, S. franciscanus* and *Patiria miniata,* rate of reduction usually decreased from that region of the cell where the nucleus was nearest the surface (Child, 1936a; Figs. 1–3, 8 and 9). This region varied with respect to attachment to ovarian tissues but was usually somewhere on the surface not in contact with ovarian tissues, though a few very early oöcytes were observed with reduction gradient decreasing from the attached pole and nucleus near that pole (Child, 1936a; Fig. 4). These may have been stages so early in oöcyte growth that other ovarian tissues still reduced more rapidly than the oöcyte. Figure 5 of the same paper with reduction decreasing in rate from the interior of the cell after staining by oxidized diazine green, is probably a case of differential injury with retardation of reduction near the cell surface; with intracellular oxidation nothing similar has ever been observed.

It is usually possible to find early oöcyte stages by gentle teasing of small portions of the ovaries, even in females with large numbers of eggs ready for fertilization. Figure 1, *a–x*, the result of repeated series of observations on all three echinoderms constituting the material of this paper, is believed to be a fairly adequate sample of the gradient patterns observed in the earlier stages of oöcyte growth and of the nuclear positions in relation to these patterns. In Figure 1, *a–l* are from *S. purpuratus, m–q* from *Dendraster* and *r–x* from *Patiria.* In most of these cells the relation to the ovarian tissue is not evident. In those which do show some indication of this relation (Fig. 1, *f, k, n, o*) the region of most rapid intracellular oxidation is at or near the "free" pole of the cell. The oöcytes differ in form and are often more or less elongated, the axis of elongation being the gradient axis or departing only slightly from it. The gradient pattern in these cells is distinct, though with apparent variation in differential in different cells. In most cases the rate of oxidation decreases rapidly from one pole with relatively slight differential in the remainder, often a half of the cell. This condition has seemed to be more distinct in *S. purpuratus* and *Dendraster* than in *Patiria,* but whether it is of real significance remains uncertain. It is apparently more frequent in the earlier stages and may perhaps indicate that the gradient is in process of developing from the high end.

Nuclear position in relation to the gradient pattern varies. In the earliest stages (Fig. 1, *a–d, r*) it may be at or near the pole of least rapid reaction, the "low" end of the gradient or in some other region of the cell, perhaps near the middle (*g, j, k, s, w*) and not always in the gradient axis. However, in the great majority of the oöcytes the nucleus is near the high end of the oxidation gradient and apparently more frequently in this position as size of the oöcyte increases, but a cell approaching full size has occasionally been seen with nucleus near the middle. These observed differences may be without real significance, but if they do represent actual physiological differences in individual cells they suggest that gradient pattern is developing in the oöcytes, that different stages of this development appear in different cells and that the nucleus gradually comes to lie near the high end of the gradient, though some variation in position may still occur in later stages.

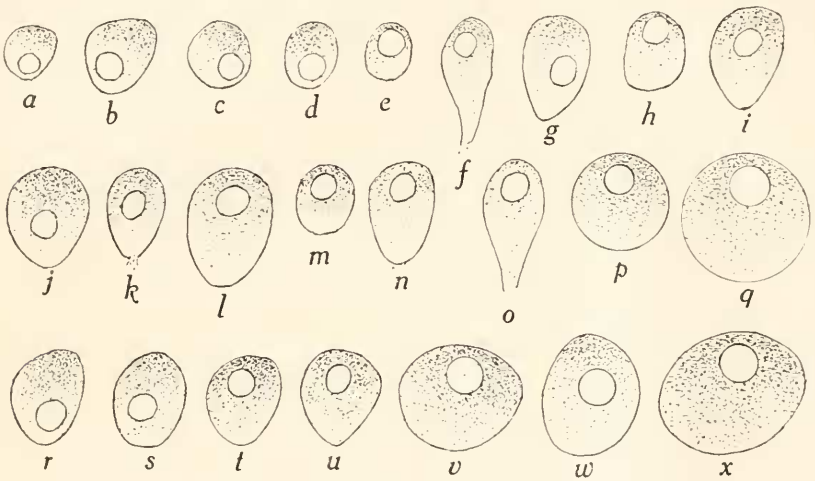

FIGURE 1. Gradient patterns of early oöcytes: *a–l, S. purpuratus; m–q, Dendraster excentricus; r–x, Patiria miniata.*

As the cell increases in size the gradient differential seems to become less distinct, *i.e.*, the lower gradient levels differ less from the higher levels than in many earlier stages, and in general the gradient appears to decrease in rate of reaction, probably as enzyme activity decreases in the later stages of growth. However, it is difficult to determine whether changes such as this are or are not significant. It is certain that the oxidation gradient pattern does become less distinct in the full grown unfertilized egg than in the early oöcyte stages.

All oöcytes of Figure 1 are placed in the figure with the region of most rapid oxidation uppermost. This involves the assumption that the gradient pattern represents a definite physiological axis, rather than a mere chance differential differing from cell to cell and without further significance. Moreover, even though these early stages alone do not provide evidence that the high ends of their gradient patterns become the apical or animal pole of the egg, comparison with later stages leaves little doubt that this is the case. It appears highly improbable that a gradient pattern involving enzymes of fundamental importance is without definite relation to oxidation patterns of later stages. It is believed, therefore, that these data con-

cerning early stages in ovarian egg development indicate a developing apicobasal physiological axis that persists and becomes the polarity of the egg and embryo.

With further growth of the oöcyte the relation of nuclear position to the high end of the indicator gradient becomes increasingly definite, though some variation still occurs. The usual nuclear position in later growth stages is indicated in Figure 2, but even in these stages an oöcyte with nucleus near the center of the cell has occasionally been seen. These cells are perhaps not in good condition. With progress of growth the indophenol reaction becomes much less rapid and the gradient differential is slight but still distinguishable with care in use of the reaction and in observation. As it approaches the end of the growth period the cell evidently becomes less active, at least so far as the oxidase or oxidases catalyzing the

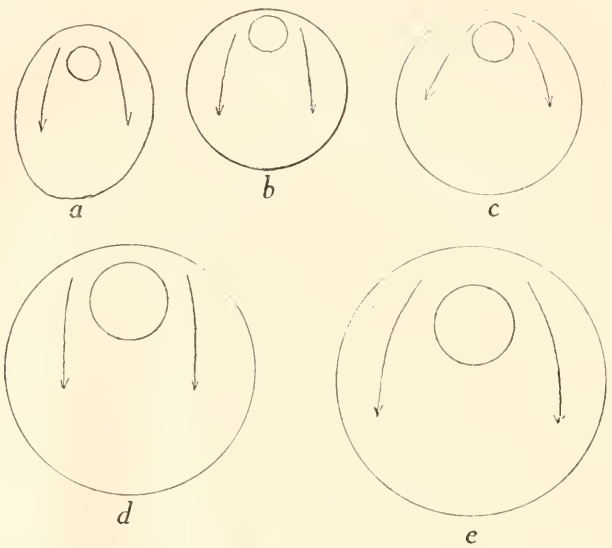

FIGURE 2. Gradient patterns of later growth stages of oöcytes: *a–d, S. purpuratus* and *Dendraster; e, Patiria.*

reaction are concerned, and preceding fertilization, or, in the case of the starfish, preceding polar body formation, it is an extremely inactive cell. In many of these cells, however, a slight gradient is still visible; in some others it has not been distinguished with certainty.

POLAR BODY STAGES

In the two echinoids the first polar body is formed in the ovary preceding spawning. In slightly teased ovaries it has been seen occasionally (Fig. 3, *a, b*). It is formed at the high end of the slight gradient. It has sometimes seemed that the gradient became slightly more distinct at the time of its formation. There is every reason to believe that the pole near which the nucleus lies in Figure 2 and the region of polar body formation are the same. It appears highly improbable that the nucleus has moved to another region of the egg after the growth period of

the oöcyte has ended. The nucleus of course becomes invisible at the time of polar body formation and afterward it is smaller but can usually be made visible by pressure (Child, 1936a; Fig. 7). The second polar bodies of the two echinoids are formed after fertilization (Fig. 3, *c, d*). The intracellular indophenol reaction becomes more rapid and the gradient more distinct following fertilization. The *Patiria* eggs were fertilized as soon as possible after extrusion from the gonads and both polar bodies formed after fertilization (Fig. 3, *e*), also with increase in distinctness of the indophenol gradient. If the *Patiria* eggs had been kept without

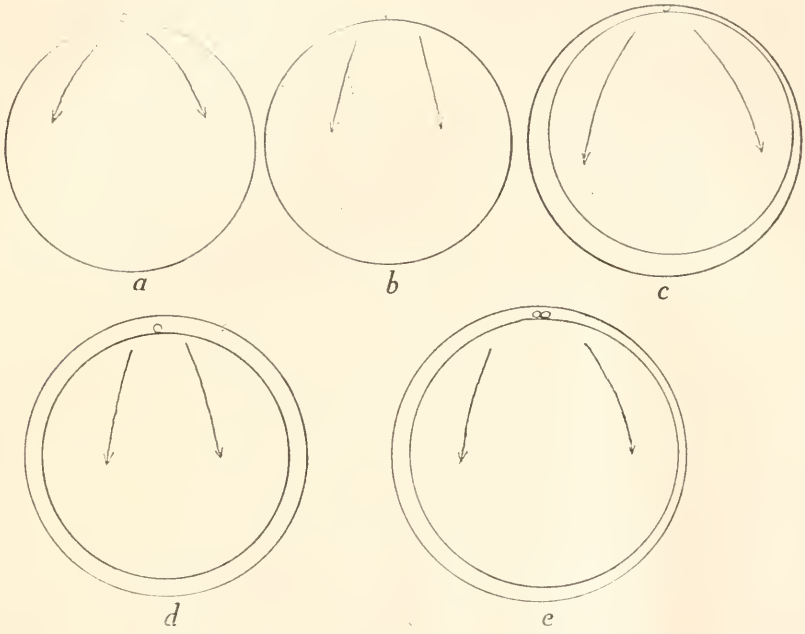

FIGURE 3. Gradient patterns of polar body stages: *a* and *b*, first polar body stage of *S. purpuratus* and *Dendraster* before fertilization; *c* and *d*, second polar body stage of these forms following fertilization; *e*, polar bodies of *Patiria*, following fertilization.

fertilization, polar body formation and activation would probably have occurred as in *Asterias* (Loeb and Wasteneys, 1912; Tang, 1931). Polar bodies formed at the pole of most rapid indophenol reaction.

EARLY CLEAVAGE STAGES

The gradient pattern in early cleavage stages of *S. purpuratus* has been rendered visible by primary intracellular oxidation of reduced methylene blue, diazine green and Nile blue sulphate as well as by the indophenol reaction, the same pattern appearing with all three procedures. Only the indophenol reaction has been used on most lots of *Dendraster* and *Patiria* with the same results in all cases. The observations on these stages have been made repeatedly on many lots of eggs during different breeding seasons from 1947 on. They have included thousands

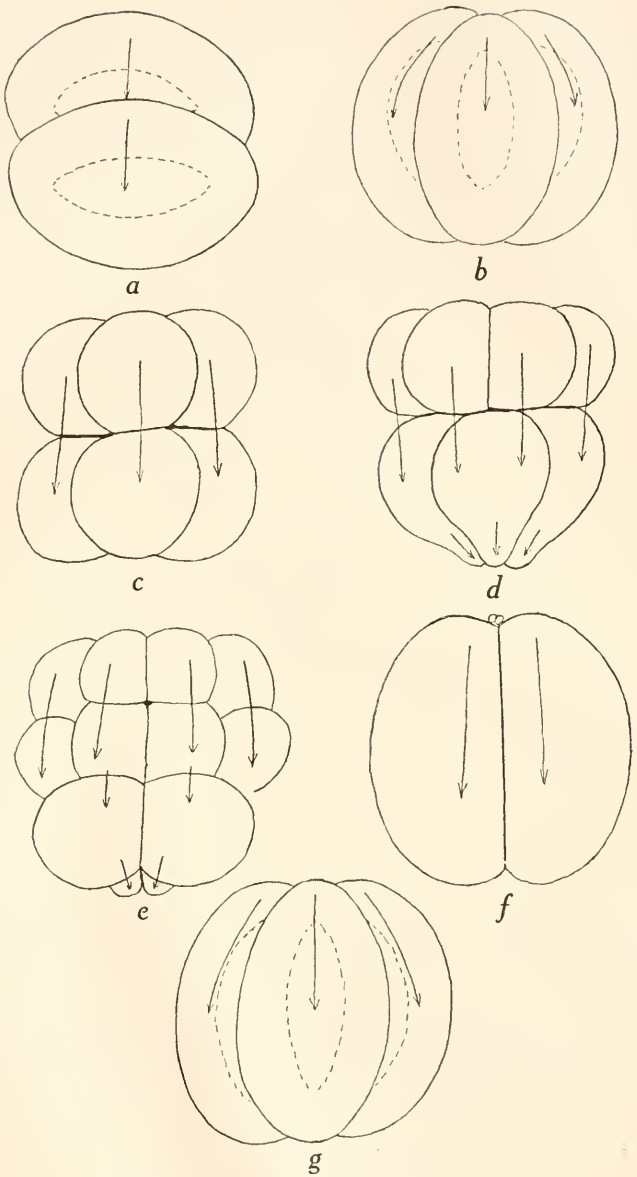

FIGURE 4. Gradient patterns of early cleavage stages: *a* and *b*, 2- and 4-cell stages of *Strongylocentrotus* and *Dendraster* with outlines of mitotic figures for next division; *c* and *d*, 8- and 16-cell stages of the two echinoids, difference between mesomeres and macromeres distinct, between macromeres and forming micromeres less distinct but visible; *e*, 24-cell stage of the echinoids, difference between mesomeres and macromeres apparently slightly more distinct in many embryos, in others essentially like 16-cell stage, micromeres still slower reaction than macromeres; *f* and *g*, 2- and 4-cell stages of *Patiria*.

of eggs under widely varied conditions of illumination. Early cleavages of *S. purpuratus* and *Dendraster* are alike, and in Figure 4, *a–e* indicate gradient pattern in both. In these forms the second polar body usually lies deep in the cleavage furrow and is often not certainly visible. For certain orientation, in Figure 4, *a,* the 2-cell stage is drawn with outlines of the mitotic figures for the second cleavage indicated. In Figure 4, *b,* the 4-cell stage, mitotic figure outlines for the third cleavage are also indicated. These are slightly nearer the pole of most rapid dye oxidation and indophenol reaction and the four apical cells of the 8-cell stage are usually slightly smaller than the basal cells (Fig. 4, *c*), though there is apparently some variation in this respect. In the 8-cell stage the differential between the apical and basal cells becomes even more distinct. In Figure 4, *d,* approaching 16 cells, the differential from mesomeres to macromeres is clearly evident and the forming micromeres show even less rapid reaction than the macromeres. At the stage of Figure 4, *d,* dye oxidation and indophenol reaction are more rapid in the apical than in the basal mesomeres with less difference from the basal mesomeres to the macromeres and the micromeres are slowest of all. Figure 4, *f* and *g,* 2- and 4-cell stages of *Patiria,* shows the same gradient pattern as the echinoids and the same as observed in an earlier paper (Child, 1944). No micromeres are formed in *Patiria* but there is a slight increase in size of blastomeres basipetally as cleavage progresses and rate of indophenol reaction decreases basipetally from the smaller cells of the apical region, as also shown in the paper of 1944, so that repetition of those data is unnecessary here. With low intracellular concentrations of oxidized dyes and indophenol it has often been possible to reduce and re-oxidize these cleavage stages without altering gradient pattern.

The Blastula and Later Stages

In the blastula of all three forms the polar gradient pattern becomes so distinct that it is clearly visible in surface view as well as in optical section. A point of interest to be noted is that the reaction at all levels is more rapid on the blastocoelar side of the cell wall, not on the external surface. In Figure 5, *a* (*S. purpuratus* and *Dendraster*) and *b, Patiria,* the arrows in the cell wall indicate the gradient in optical section, the other arrows the gradient in surface view. The differential from the blastocoelar surface outward is indicated only at the apical end by the optical section arrows drawn from the blastocoelar surface. Thus far it has not been possible to distinguish with certainty a ventrodorsal gradient in blastulae. In some blastulae intracellular oxidation seemed to be slightly more rapid on one side but even if this is actually the case it is not certain that the more rapid side is ventral, as it is in later stages, or whether any probable differential is due to some incidental factor. Probably a ventrodorsal pattern is present at this stage and even earlier but it is either too slight to become distinctly visible in intracellular oxidation or may conceivably differ in character from the ventrodorsal gradient of later stages. In Figure 5, *c,* the gastrula of *S. purpuratus* and in *d* the gastrula of *Dendraster* with somewhat longer apicobasal axis are outlined. Polar and ventrodorsal gradients are both clearly visible in the ectoderm. In the invaginating entoderm a new longitudinal gradient has arisen with rate of oxidation decreasing from the tip. In Figure 5, *e,* the pre-pluteus or early pluteus of the echinoids is outlined in somewhat oblique optical section from the side, with the gradient pattern indicated. The

ectodermal polar gradient is becoming more distinct as the oral lobe develops from the apical region. The ventrodorsal gradient is also more distinct than in earlier stages, and a new gradient decreasing from the tip of each developing anal arm is now present. In stages *c*, *d* and *e* the ectodermal pattern is sufficiently distinct to be visible in surface view and optical section. The entodermal differential has also increased. As regards the intracellular indophenol pattern of the later stages of *Patiria* it is unnecessary to repeat here the data recorded in the earlier paper (Child, 1944).

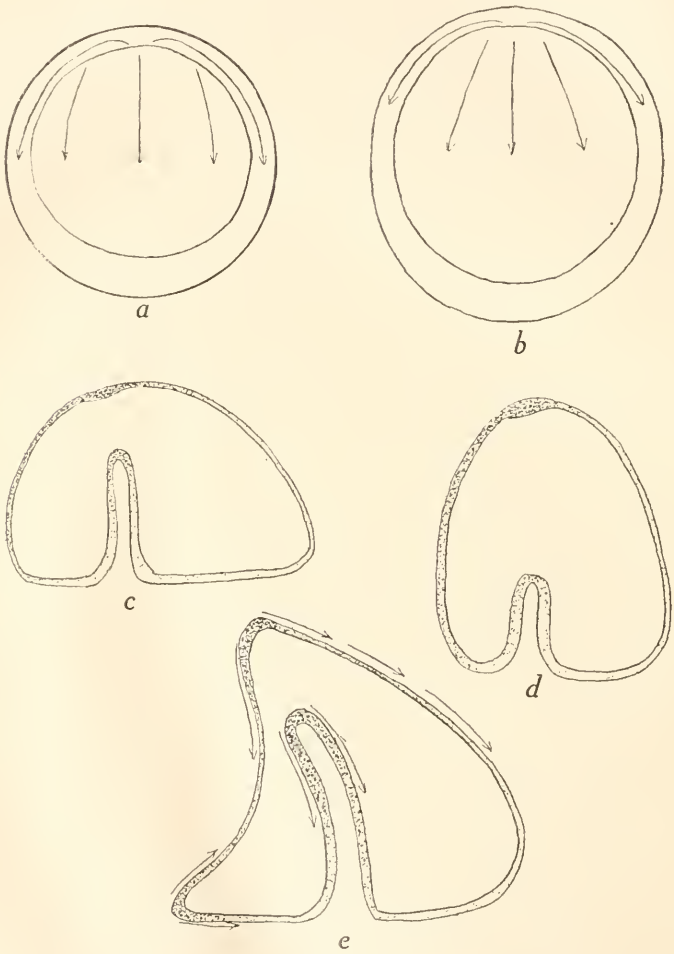

FIGURE 5. Gradient patterns of later stages; differential becomes increasingly distinct during blastula stage. Arrows in cell wall indicate gradient in optical section, other arrows, gradient in surface view. *a*, the two echinoids; *b*, *Patiria; c*, gastrula of *S. purpuratus; d*, gastrula of *Dendraster*, apicobasal, ventrodorsal and entodermal gradients indicated in both; *e*, somewhat oblique outline from side of early pluteus of echinoids, gradient of developing oral lobe in apical region, ventrodorsal gradient, gradient in developing anal arm and gradient in entoderm indicated.

Without feeding, the echinoid plutei gradually die of starvation. During the later stages of their lives intracellular oxidation of reduced dyes and of indophenol reagents becomes less rapid and gradient differentials decrease. Even while they are still to some degree motile they show little evidence of oxidase activity and little or no gradient pattern. Starving larvae of *Patiria* show very similar decrease in gradient pattern and of oxidase activity.

Discussion

An intracellular oxidation gradient pattern of indophenol reagents and of various reduced redox dyes is present through ovarian development of the oöcyte and through embryonic and larval development. Intracellular oxidation is more effective than reduction in rendering visible slight differentials; staining by oxidized dyes preceding reduction may itself decrease differentials. The differential developmental modifications by external agents show the very definite relations of morphogenesis to this gradient pattern. In the early oöcytes the gradient pattern is very distinct but becomes less so as developmental activity of the oöcyte decreases in later stages. The course of oöcyte development suggests that the cell acquires a cytoplasmic gradient pattern from a differential in ovarian environment, perhaps an oxygen differential, and that the nucleus gradually attains a position near the most active region of this gradient, polar bodies form there and the gradient becomes the basis of physiological polarity in the embryo and larva.

A ventrodorsal pattern of some sort is probably present long before it becomes distinguishable by means of the indicators. It is perhaps of interest to note that in the growth of the ovaries the small finger-like outgrowths show an indicator oxidation gradient decreasing from their tips. Perhaps the oöcyte is subjected from its earliest stages to an ovarian differential at right angles to the polar differential. If this determines a differential in the cell it may be either too slight in early stages to appear, even in intracellular oxidation of indicators, or may not yet have developed a differential in oxidase activity.

The present paper makes it necessary to refer again to the failure of Lindahl and Holter (1940) to find any significant difference in oxygen uptake in apical (animal) and basal (vegetal) halves of developmental stages of *Paracentrotus lividus* and to the somewhat later assertion of Lindahl (1942a, 1942b) that the data of Child concerning reduction gradients in a sea urchin cannot be "valid" because no differential in oxygen uptake was found in the separated halves of embryos. This matter was discussed in an earlier paper (Child, 1944) and only certain points need be noted here. Lindahl's assertion concerning validity seems not entirely justified on the basis of his and Holter's purely negative evidence and in the face of positive evidence to the contrary. These authors do not mention any attempt to determine whether a much larger number of embryo halves than they used would show a differential. Also an error in one of their tables invalidates certain of their conclusions. Moreover, they admit an error of 10 per cent. It has never been maintained that indicator gradients must always parallel regional differentials in oxygen uptake, though they are parallel in various organisms. It is conceivable that separation of the echinoderm stages into parts may decrease or obscure regional differences in oxygen uptake, particularly if these are slight. Possibly also other factors may sometimes be involved in oxygen uptake than those determining indi-

cator patterns. The indicator methods have certain advantages over even Cartesian diver methods of determining oxygen uptake. Although they are not quantitative they are more delicate than any other method and make directly visible very slight regional differentials even in very small cells without isolation of parts. There can be no question concerning validity of the data of the present paper. With care in use of indicators and in observation, these gradient patterns can be seen by anyone not color blind and with fairly good visual perception of slight differences in depth of color. Also, with a little practice ability to distinguish slight differentials with a high degree of certainty increases.

The critic may question whether the observed gradient patterns are actually the same in different oöcytes and at different stages or whether they are merely chance differentials. In those early oöcytes showing evidence of relation to ovarian tissues the most active ends of the gradients are at or near the opposite pole. The increasingly definite relation of the nuclei to these more active gradient regions, the formation of polar bodies there and the relation of gradient patterns to physiological axes indicate their significance. In this connection it is of interest that, according to Tennent (1931), the first polar body of the sea urchin, *Mespilia globulus,* forms at the free pole of the ovarian cell.

Since this paper is concerned with gradient patterns, the occasion is taken to refer to the interesting experiments on alteration of ventrodorsality by subjection of *Dendraster* embryos at the 8-cell stage to a concentration gradient of various agents (Pease, 1941, 1942a, 1942b). Some of the agents used are known or believed to inhibit certain enzyme systems, with perhaps still other effects; actions of some others are perhaps less well known. The effective agents determined the most inhibited region as dorsal and the less inhibited became ventral with a frequency sufficient to demonstrate a positive determining action. Indicators do not show ventrodorsality in early embryonic stages, though it is doubtless present in some degree or form (see also Hörstadius and Wolsky, 1936). When it becomes visible as an indicator gradient it decreases from the ventral side dorsipetally. There is no distinguishable difference between right and left sides in early stages. Pease speaks repeatedly of his determination of bilaterality, although he actually determines ventrodorsality. Since the embryo has three spatial dimensions and since there is no evidence of asymmetry in the stages concerned, bilaterality is only an incidental consequence of determination of ventrodorsality.

In use of various agents Pease found that inhibition of cleavage and determination of ventrodorsality were not necessarily associated in action of a particular agent. To quote him concerning this point: "This leads to the conclusion that the bilateral (sic) determination is not dependent on a vague metabolic gradient but is rather dependent upon a specific enzyme system or linked systems" (Pease, 1941; p. 399). And again: "Bilateral (sic) determination by chemical concentration gradients is not dependent upon general metabolic gradients because this determination can be separated from differential cleavage inhibition" (Pease, 1942b; p. 352). The meaning of these statements is not clear. Do they mean that the ventrodorsality which he has determined is different in physiological character from the ventrodorsality of the uninhibited embryo? If this is the meaning, two different sorts of ventrodorsality can result in the same course of ventrodorsal development. This appears highly improbable. Moreover, cleavage, though to some degree correlated with gradient pattern, also unquestionably involves various activities which have

little or no relation to that pattern. Some agents may inhibit these without any effect on ventrodorsality. There is every reason to believe that the experimentally determined ventrodorsality is the same as ventrodorsality in uninhibited development. Examination and counts of the material were made at the late gastrula or "prism" stage, but the question whether the experimental ventrodorsality is so completely determined that the fully developed pluteus form is attained or whether there is some differential inhibition, is not considered by Pease.

Pease also says: "The extreme sensitivity of the bilateral (!) determination to concentration gradients suggested that respiratory mechanisms might be involved (Pease, 1941). The further successful experiments with azide demonstrated with little probability of error that an oxidation system passing through a heavy metal catalyst is important in this determination" (Pease, 1942, pp. 352–3). The following pages of the summary of his paper are largely concerned with hypotheses concerning the enzyme systems involved in the determination of ventrodorsality. He seems not to be aware that according to present conceptions the action of redox indicators depends on an oxidase or oxidases and on one or more dehydrogenases. Moreover, various agents effective in determining differential developmental modifications are known to be oxidase or dehydrogenase inhibitors, or more or less general inhibitors of enzyme activity. If these respiratory enzyme systems are as important in the ventrodorsal determination as Pease maintains, there can be no doubt that the experimentally determined ventrodorsal gradient is the same physiologically as the gradient made visible by the redox indicators in uninhibited development. Incidentally, although Pease is evidently convinced that he has determined bilaterality, he does not consider how the external gradient could determine a bilaterality at a right angle to its own differential and without distinguishable difference on the two sides.

Pease's experiments suggest that a certain degree of physiological dominance of the ventral region, the high end of the ventrodorsal gradient pattern according to the redox indicators, over the less active dorsal region is present in unaltered development. With inhibition of the dominant ventral region, the dorsal region becomes more or less physiologically isolated, its activity increases and it develops as a ventral region. The experiments suggest further that in early developmental stages the difference between ventral and dorsal is at least very largely, if not entirely, quantitative.

The indicator gradients and the differential developmental modifications also suggest that in the early development of the echinoderms and of many other organisms regional differences in at least certain enzyme systems are predominantly or entirely quantitative, and that regionally specific differences gradually arise. Does not differentiation consist largely in regional localization of certain enzyme systems, while certain others remain less definitely localized? Apparently oxidases and dehydrogenases, or certain of them, are among those which usually remain rather generally distributed. In many organisms quantitative differentials in indicator patterns persist throughout life.

Most of our knowledge, as distinguished from hypotheses concerning enzymes, has been obtained by destruction of living protoplasms. We know little concerning the internal relations of enzyme systems in living, apparently uninjured organisms. The indicator methods are not quantitative but they give information which at present can be obtained in no other way. Also the differential developmental

modifications give information concerning the significance of the indicator gradient patterns in developmental morphogenesis, and it appears probable that with further progress and refinement in use of external agents, they will give information concerning other than the respiratory enzyme systems.

In view of the present interest in enzyme systems it seems desirable to call attention to another aspect of action of an external agent on living organisms. In a system in which continuous correlated change is occurring, the rate of that change, in general terms the activity of the system, may be an important factor in determining its sensitivity or susceptibility to an external agent. Insofar as quantitative differences in activity are characteristic of the system or its parts, susceptibility to an external agent which retards, inhibits or otherwise alters any essential quantitative factor of the system, and so alters the system, will vary with the activity. The effect of the agent will occur more rapidly and in greater degree in the more active than in the less active system or part, irrespective of the particular character of the action of the agent. In the echinoderm eggs and embryos regional quantitative differentials in at least certain of the correlated changes characteristic of the gradient pattern are present, though not necessarily the only regional differences present. Susceptibility to any agent which acts on an essential factor of this quantitative differential will be greater in the more active than in the less active gradient levels. In other words, the action of the agent will be differential in relation to the gradient pattern; by an inhibiting agent the more active "higher" gradient levels will be more inhibited than less active levels, whatever the particular factor on which the agent acts. Also, recovery from temporary action will be more rapid and more complete at more active than at less active levels, provided action of the agent has not become irreversible, and equilibration or development of tolerance and acclimation will show the same relation to the gradient pattern. This relation of susceptibility to external agents and activity accounts for the determination of similar differential developmental modifications by many external agents which certainly do not all act in the same way on a protoplasm. And conversely, these similar modifications by different agents constitute evidence that in the echinoderms and various other organisms quantitative differentials are important factors in gradient pattern. This relation of susceptibility to activity by no means excludes the possibility that certain agents may provide evidence of specific regional differences in effect; perhaps some modifications of echinoderm development are suggestive of such effects.

The relation of susceptibility to activity holds for inorganic systems with quantitative differences in activity as well as for living organisms. For example, a rapid stream or a rapidly moving automobile is more susceptible to a sufficient degree of disturbance of any kind than a slow stream or car, and recovery from less extreme temporary disturbance and equilibration to a slight disturbance show the same relation to activity.

In conclusion, on the basis of evidence at present available it appears beyond question that a gradient pattern, primarily predominantly or entirely quantitative, is an essential factor in echinoderm larval development. Moreover, this pattern provides a physiological basis for activation of different genes in different cells or cell groups, *i.e.*, for differentiation. When this pattern is altered experimentally the course of morphogenesis is altered; when gradient pattern is obliterated, development and differentiation do not occur unless a new gradient is determined by an external agent, as has been done in certain organisms.

SUMMARY

1. With progress in the use of redox indicators, it has been found that slight gradient differentials become more distinctly visible by intracellular oxidation of redox dyes, reduced by essentially non-toxic sodium hydrosulphite, and of low concentrations of indophenol reagents than in reduction of oxidized dyes. Staining by oxidized dyes preceding reduction may itself decrease slight differentials.

2. By means of intracellular oxidation of reduced dyes and indophenol reagents a gradient pattern has been rendered directly visible from early oöcyte to larval stages. The evidence indicates that the gradient of the early oöcyte becomes the polar gradient of the egg and embryo. In early oöcyte stages, position of the nucleus varies, but as oöcyte growth progresses it comes to lie near the pole of most rapid intracellular oxidation, the polar bodies form there and this becomes the apical (animal) pole of egg and embryo.

3. The ventrodorsal gradient pattern becomes visible in the gastrula, perhaps in the late blastula, but is undoubtedly present earlier, probably with a differential too slight to be visible or in another physiological condition. Other gradients appear in the further course of development.

4. Experiments of Pease on determination of ventrodorsality in *Dendraster* by gradients of inhibitory agents are discussed in relation to redox gradient pattern.

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